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(54) Title: NOVEL TREATMENTS FOR ALLERGIC DISEASES

(57) Abstract

The present invention provides novel peptides for treating allergic diseases such as celiac disease. The peptides bind MHC glycoproteins associated with celiac disease and block T cell activation associated with the disease. The peptides may block MHC-antigenic peptide complex formation. Alternatively, the peptides may be T cell antagonist peptides and form inhibitory complexes which competitively inhibit binding between MHC-antigenic peptide complexes which induce activation of T cells associated with celiac disease. Methods for identifying peptides of the present invention are also disclosed.

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NOVEL TREATMENTS FOR ALLERGIC DISEASES

BACKGROUND OF THE INVENTION

The present invention relates to allergic diseases such as celiac disease and to materials useful in the diagnosis and treatment of these diseases. In particular, it relates to novel peptides recognized by pathogenic T cells associated with the disease, e.g, celiac disease.

Celiac disease, also called non-tropical sprue, is a disorder characterized by malabsorption, abnormal small bowel structure and intolerance to gluten, a protein found in wheat and wheat products. The incidence of celiac disease is difficult to estimate because the severity of the disease varies greatly and individuals with the disease may have no overt symptoms. Seventy percent of the cases reported are women.

The incidence of the disease among siblings is many times higher than that in the general population, and it has been suggested that the disease may be inherited. Celiac patients have an increased frequency of certain MHC haplotypes, particularly of the HLA-B8 and HLA-Dw3 types. The HLA-B8 haplotype has been found in 85-90% of celiac patients as compared with 20 to 25% in normal subjects.

It has been suggested that gluten or gluten metabolites initiate an immunological response in the intestinal mucosa. Gluten, which comprises gliadins and glutenins, is a major component of the wheat endosperm. The alcohol soluble gliadin fraction of gluten has been clearly demonstrated to activate celiac disease. In rye, barley, and oats, the alcohol soluble proteins associated with the activation of disease are termed secalins, hordeins, and avenins, respectively. For a general review of celiac disease see, Kagnoff, Gut and Intest. Immunol., 8:505 (1988), which is incorporated herein by reference.

There are currently no reliable methods for treating celiac disease. Diagnostic procedures typically require gut biopsies. The mainstay of treatment is imposition of gluten-free diet. Although most patients improve within a few weeks of starting the diet, avoiding ubiquitous wheat products, such as bread, pasta, and the like, can obviously be burdensome. Moreover, wheat is frequently used as an extender in many processed foods, such as salad dressings, canned vegetables and soups, ice cream, and candy bars.

The invasive diagnostic procedures and inadequate treatments presently available demonstrate the urgent need for agents to treat celiac disease. Such agents should also be economical to produce and possess favorable pharmacologic properties for example a relatively long half-life, thereby facilitating lower dosages and/or less frequent administration.

SUMMARY OF THE INVENTION

The present invention relates to peptides useful for diagnosing or treating celiac disease. The peptides bind an MHC molecule on an antigen presenting cell associated with celiac disease and thereby inhibit activation of pathogenic T cells. Typically, the peptides inhibit binding of an antigenic peptide to the MHC molecule. Alternatively, the peptides may be T cell antagonist peptides and bind to the MHC molecule to form an inhibitory complex.

The peptides typically consist of between about 5 and about 25 amino acids and may comprise one or more amino acid or peptide bond mimetic. The peptides may also comprise a D-amino acid.

The present invention also relates to the discovery that antigen presenting cells expressing DR7 or DR53 MHC glycoproteins are involved in antigen presentation in celiac disease.

The invention further relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and the peptides described above. Methods of treating celiac disease comprising administering to a patient a therapeutically

effective dose of the pharmaceutical compositions are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents data showing that pulsing target T cells with T cell receptor antagonist peptides does not inhibit the proliferative response, while incubation of antigen presenting cells with the peptide induces dose-dependent inhibition of T cell proliferation.

Fig. 2 presents data showing that preincubation of T cells with antigenic peptides results in induction of T cell unresponsiveness (anergy) but preincubation with T cell antagonist peptides does not.

Fig. 3 demonstrates that T cell antagonist peptides (unlike antigenic peptides) do not induce IL-2 production in T cells as measured by growth of the IL-2 dependent HT-2 cell line.

Fig. 4 demonstrates that MHC complexes comprising T cell antagonist peptides do not induce detectable increases in inositol phosphate production.

Fig. 5A presents data showing that peptides unrelated to the antigenic peptide act as inhibitors of antigen presentation only in the classical MHC competition assay, but are devoid of any activity in a prepulse assay.

Figs. 5B and C5 show that in prepulse assays T cell antagonist peptides act as potent inhibitors of target T cell proliferation but have no effect on the proliferation of non-target T cells.

Fig. 6 shows proliferative dose response of JR and RCL, alpha gliadin T cell lines. T cell lines were testes in the proliferation assay as described in the Materials and Methods, using autologous EBV as APCS. JR (open circle) and RCL (closed circle) were derived from a normal donor and a CD patient, respectively.

Fig. 7 shows both JR and RCL lines are DR-restricted. mAb inhibition studies were performed using 10 µg/ml of purified anti-class II mabs specific for various isotypes [LB3.1 (closed square) anti-DR; B27.11 (lined square) anti-DP; and 11B.5 (half-tone square) anti-DQ]. The rabies

glycoprotein 285-299 DR7-restricted clone C25 and the alloreactive DQ2.7-restricted T cell clone were also used as controls. Positive (100% inhibited) controls and antigen doses used were: JR, 100 μg/ml; RCL, 100 μg/ml; C25 0.1 μg/ml; and JS87 1 x 10^3 irradiated DR7 EBV line.

Figs. 8A and 8B show genetic restriction of JR and RCL T cell lines. JR (Fig. 8A) and RCL (Fig. 8B) T cell lines were tested for their capacity to proliferate in response to autologous or homozygous EBV lines or DR-transfected fibroblasts, in presence (closed bars) or absence (open bars) of 100 μg/ml purified whole alpha gliadin.

Figs. 9A-9C show differential antigen-processing capacity of EBV lines and DR-transfected fibroblasts. Proliferation of the alpha gliadin-specific clones (Figs. 9A and 9B) or the PPD-specific line VIII (Fig. 9C) was measured in response to a dose range of antigen, utilizing either irradiated EBV lines (closed circles) or irradiated DR7-transfected fibroblasts (closed squares) as APCS.

Fig. 10A and 10B show proliferation of RCL cloned T cells in response to a dose range of alpha gliadin peptides presented by EBV lines or DR-transfected fibroblasts. Proliferation of RCL T cells in response to a dose range of alpha gliadin (AGL) 1-20 (10A) or alpha gliadin (AGL) 1-8 (10B) was measured. The APC types used were: irradiated DR-transfected fibroblasts (closed squares); irradiated homozygous EBV lines (closed circles); and fixed homozygous EBV lines (open circles).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compositions of the invention comprise peptides recognized by T cells associated with the disease. The peptides can be used, linked to the appropriate molecule or carrier, for the diagnosis (e.g., using intradermal reaction skin tests) of the disease. The peptides can be used for treatment by 1) vaccination 20 induction of tolerance, or 3 inhibition by substituting novel peptides for the native peptide to inhibit the immune response. The sequence of the peptides is preferably based on an antigen derived from wheat

gluten, preferably from the gliadin fraction. The peptides and analogs of interest can be tested for the ability to inhibit T cell activation using the protocols described below.

It should be understood that the nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxy group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxy-terminal groups, although not specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. In the present application, the L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G.

As is explained in detail below, the invention provides the ability to inhibit antigen specific immune responses associated with particular T cell clones. This invention includes the discovery of the antigenic (or autoantigenic) peptides recognized by pathogenic T cells when the antigenic peptide is unknown. The two major classes of immune response are mediated by different classes of lymphocytes, B cells, which produce antibodies, and T cells, which are responsible for cell-mediated immunity. Unlike B cells, T cells see antigen only when bound to MHC glycoproteins on antigen presenting cells.

MHC molecules are heterodimeric glycoproteins expressed on cells of higher vertebrates and play a role in immune responses. MHC glycoproteins are divided into two groups, class I and class II, which differ structurally and functionally from each other. In general, the major function of MHC molecules is to bind antigenic peptides and display them

on the surface of cells. These peptides result from an antigen presenting cell (APC) processing an antigen into peptide fragments, which can be as short as 8 to 20 amino acids.

Class I MHC molecules are expressed on almost all nucleated cells and are recognized by cytotoxic T lymphocytes, which then destroy the antigen-bearing cells. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular antigenic peptide that is displayed. Engagement of the T cell receptor induces a series of molecular events characteristic of cell activation, such as, increase in tyrosine phosphorylation, Ca^{++} influx, PI turnover, synthesis of cytokines and cytokine receptors, and cell division (see, Altman et al., Adv. Immunol. 48:227-360 (1990), which is incomparable herein by reference). For a general discussion of how T cells recognize antigen see Grey, H.M., et al., Scientific American pp 56-64, (November, 1989) and Fundamental Immunology, 2d Ed., W.E. Paul, ed., Ravens Press N.Y. 1989, both of which are incorporated herein by reference.

The present invention provides novel treatments for any condition involving unwanted T cell reactivity, such as foreign infectious diseases that can cause immunopathology (e.g., lyme disease, hepatitis, LCMV, post-streptococcal myocarditis, or glomerulonephritis). In addition, food hypersensitivities such as celiac disease and Crohn disease as well as other allergic diseases associated with particular histocompatibility haplotypes. For a review of allergic diseases suitable for treatment using the methods of the present invention see, O'Hehire, et al. Ann. Rev. Immunol. 9:67-95 (1991), which is incorporated herein by reference. Although the treatment of celiac disease is disclosed in detail, it will be understood that the same general approach can be used for other allergic diseases.

As mentioned above, the antigen associated with

celiac disease is the gliadin fraction of gluten. Gliadins are single polypeptide chains that range in molecular weight from 30,000 to 75,000 daltons. They have a low charge and a high glutamine and proline content. These proteins have been categorized into four major electrophoretic fractions: alpha gliadins, beta gliadins, gamma gliadins and omega gliadins. When alpha gliadins from suitable wheat varieties are ultra-centrifuged, a precipitate of aggregatable alpha gliadins termed alpha gliadin is formed. This major alpha gliadin fraction is known to activate disease. The complete primary amino acid sequence of alpha gliadin has been determined from amino acid sequencing and this and other gliadin sequences have been deduced from sequences of cDNA clones. Bartels et al., Nucleic Acids Res. 11:2961 (1983); Kasarda et al., Proc. Nat'l. Acad. Sci. (USA) 81:4712 (1984); and Rafalski et al. EMBO J. 3:1409 (1984), which are incorporated herein by reference.

As mentioned above, celiac disease is strongly associated with HLA antigens. Initially, the association was noted to be with the HLA Class I marker HLA-B8. Later studies suggested stronger association between celiac disease and HLA Class II D markers. A strong association is believed to be with the HLA-DR3 (DRw17) and HLA-DQw2 haplotypes. In molecular terms, the strongest association is with expression of the DQ2.3 haplotype. Tiwani et al., HLA and Disease Associations (Springer, NY, 1985), which is incorporated herein by reference. The present application, however, provides evidence that gliadin-specific T cells are DR restricted.

Peptide fragments from gliadins capable of binding the appropriate MHC molecule can be identified using a number of methods well known to those of skill in the art. For instance, PCT application No. WO 92/02543 (which is incorporated herein by reference) describes assays for measuring binding to a desired MHC molecule. The ability of the peptide to activate the appropriate T cell can determined using in vitro assays using antigen presenting cells expressing a preselected MHC molecule. Suitable assays for this purpose are described in the Example section below.

The peptides identified by these methods are referred

to herein as "antigenic peptides." Antigenic peptides bind the appropriate MHC glycoprotein to form, what are referred to herein as, "MHC-antigenic peptide complexes", which then induce activation of the target T cells. For instance, antigenic peptides associated with celiac disease comprise that portion of an antigen (e.g., alpha gliadin) that binds the appropriate MHC glycoprotein (e.g. DR7) and induces activation of a T cell associated with celiac disease.

A large number of cells with defined MHC molecules, particularly MHC Class II molecules, are known and readily available to the artisan from, for instance, the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A., which is incorporated herein by reference. T cell clones associated with celiac disease may be obtained, for instance, from peripheral blood lymphocytes derived from healthy volunteers and celiac disease patients. Clones which proliferate in the presence of peptide fragments of the antigen can then be identified.

Analogs of the antigenic peptides identified above are then screened for the ability to bind the appropriate MHC molecule and inhibit T cell proliferation. Peptides identified in these assays have immunosuppressive activity in that they can compete with the antigenic peptide for MHC binding. The present invention provides further assays in which the peptides are also tested for their ability to competitively inhibit binding of MHC-antigenic peptide complexes that induce T cell proliferation.

Alternatively, using recombinant DNA techniques, modified antigenic proteins (e.g., alpha gliadin) can be generated and screened in in vitro assays using live antigen presenting cells which process the protein and present the blocking peptide bound to the appropriate MHC glycoprotein (e.g., DR7). The proteins need not be antigenic proteins, but may be other unrelated proteins comprising sequences recognized by T cells associated with the disease. These proteins can also be screened for the ability to block celiac disease in celiac patients. The modified gliadin proteins identified in

this manner can block T cell activation by either inhibiting binding of antigenic peptides to MHC molecules or by competitively inhibiting binding of MHC-antigenic peptide complexes to T cells. In yet another approach, deletion mutants of the protein antigen are screened in celiac patients for the ability to abolish pathogenicity. In this manner, amino acid sequences responsible for induction of the T cell activation are identified. Methods for generating and expressing modified proteins using in vitro mutagenesis are well known (see, Sambrook et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, which is incorporated herein by reference).

The peptides or protein antigens identified by these assays comprise sequences necessary for recognition by the appropriate MHC allele (the agretope) and thus inhibit binding of the natural antigen to the MHC molecule. The peptides may also comprise sequences necessary for recognition by the appropriate T cell receptor (the epitope). Ideally, multiple changes are introduced into the peptides so that they comprise sequences recognized by more than one T cell clone. Thus, a single peptide inhibits polyclonal responses to an antigen. The sequences on the peptides recognized by the T cell receptors, however, are sufficiently different from the wild-type epitope, so that, although binding occurs, T cell activation (e.g., proliferation) does not occur. The ability of the peptides, once bound to MHC glycoproteins, to bind T cell receptors without inducing T cell activation is referred to here as T cell antagonism. Such peptides are referred to as "T cell antagonist peptides."

As used herein, "antagonists" are compounds which reverse or inhibit the physiological effect of a ligand or exclude binding of the ligand to a receptor. An antagonist competes directly or indirectly with the ligand (e.g., MHC-antigenic peptide complex) for the receptor (e.g., a T cell receptor) and, thus, reduces the proportion of ligand molecules bound to the receptor. Typically, an antagonist is the topographical equivalent of the natural ligand and will compete

directly with the ligand for the binding site on the receptor. Such a compound is referred to here as a "mimetic." A ligand mimetic is a molecule (or complex of molecules) that conformationally and functionally serves as substitute for the natural ligand recognized by a receptor.

"Inhibitory complexes" of the present invention comprise T cell antagonist peptides bound to the appropriate MHC molecule. These complexes competitively inhibit binding by MHC-antigenic peptide complexes. As used herein "competitive inhibition" refers to the ability of complexes comprising peptides of the present invention to compete directly or indirectly with MHC-antigenic peptide complexes. In particular, the antagonistic MHC-peptide complexes of the invention act as classical competitive inhibitors in that their inhibitory effect can be overcome by a sufficiently high concentration of the complexes which induce proliferation.

The data presented below shows that in the case of the T cell receptor (as in the case of many other membrane receptors), besides binding followed by activation (natural ligands or agonists) and no binding (obviously not followed by any activation; non-ligands), an intermediate state is attainable. This state entails engagement of the receptor by an agonist which does not trigger the biological response of the ligand. In this case, the antagonist analog comprises the MHC-peptide complex.

Peptides analogs of an antigenic peptide may be determined by, e.g. synthesizing overlapping peptides, and/or employing N-terminal or C-terminal deletions (truncations) or additions. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase Peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein. Mutant protein antigens which are processed by antigen presenting cells to form blocking peptides are recombinantly produced using suitable expression systems well known to those of skill in the art.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to inhibit celiac disease. For instance, the peptides can be modified by extending, decreasing or substituting in the compound's amino acid sequence, e.g., by the addition or deletion of amino acids on either the amino terminal or carboxy terminal end, or both, of the sequences disclosed above.

The peptides of the invention can be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L-α-amino acids, or their D-isomers, but may include non-protein amino acids as well, such as β-γ-δ-amino acids, as well as many derivatives of L-α-amino acids.

Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding. For instance, with peptide I, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various DR molecules and T cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such

substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, stearic and charge interference which might disrupt binding.

The effect of single amino acid substitutions may also be probed using D-amino acids. Modifications of peptides with various amino acid mimetics or D-amino acids, for instance at the N- or C- termini, are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al., Eur. J. Drug Metab. Pharmacokin. 11:291-302 (1986); Walter et al., Proc. Soc. Exp. Biol. Med. 148:98-103 (1975); Witter et al., Neuroendocrinology 30:377-381 (1980); Verhoef et al., J. Endocrinology 110:557-562 (1986); Handa et al., Eur. J. Pharmacol. 70:531-540 (1981); Bizzozero et al., Eur. J. Biochem. 122:251-258 (1982); Chang, Eur. J. Biochem. 151:217-224 (1985), all of which are incorporated herein by reference.

The peptides may also comprise isosteres of two or more residues in the antigenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the stearic conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, , Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. VII (Weinstein ed., 1983), which is incorporated herein by reference).

The peptides of the present invention which bind to MHC molecules and inhibit or block MHC restricted antigen-specific T cell activation will generally comprise at least 5 amino acid residues or the conformational equivalent thereof, more usually at least 6, more often at least about 8 and frequently at least about 13 residues long, and usually will

not exceed up to about 28 residues or the equivalent thereof in length, more typically 14 residues or less, and preferably no more than about 18 to 25 amino acid residues or the equivalent thereof in length. The approximate length of the peptide should generally not exceed that which may be accommodated by the binding domain of the selected MHC molecule.

The biological activity of the peptide, i.e., the ability to inhibit antigen-specific T cell activation (typically, proliferation of T helper cells), is assayed in a variety of systems. The assays are designed to detect the ability of the peptides to form MHC-peptide complexes which competitively inhibit selective binding of MHC-antigenic peptide complexes to T cell receptors on target T cells. Selective binding as used herein refers to specific recognition by one molecule (e.g., the T cell receptor) of another molecule or complex of molecules (e.g., the MHC-peptide complex) by the spatial or polar organization of a recognition determinant on the second molecule or complex of molecules.

For instance, the assays are carried out by incubating the peptide with an antigen presenting cell of known MHC expression, and a target T cell clone of known antigen specificity (i.e., T cells specific for an antigen associated with celiac disease) and MHC restriction, and the antigenic peptide itself. The assay culture is incubated for a sufficient time for T cell proliferation, such as four days, and proliferation is then measured using standard procedures, such as pulsing with tritiated thymidine during the last 18 hours of incubation. If T cell hybridomas are used in the assay, interleukin-2 production is detected. The percent inhibition, compared to the controls which received no inhibitor, is then calculated. The experiment is repeated with a non-target T cell clone. As used herein, a non-target T cell is one with the same MHC restriction as the target T cell, but which recognizes an a different antigen, antigenic peptide, or epitope.

Peptides which inhibit target T cell activation to a greater extent than they inhibit non-target T cell activation are selected. Peptides identified by this method comprise

sequences specifically recognized by the target T cells, but not by the non-target T cells. Thus, they are able to competitively inhibit binding between the T cells and antigen presenting cells in one assay but not the other.

A second exemplary assay involves pre-pulsing antigen presenting cells with a suboptimal dose of antigenic peptides. A suboptimal dose is selected such that less than about 1% of the available MHC glycoproteins are occupied by antigenic peptides. The pre-pulsed antigen presenting cells are then incubated with the peptides to be tested and the appropriate target T cell clones. Inhibition of T cell activation is then measured as described above. Since the antigen presenting cells are pre-pulsed with antigenic peptides, competition for T cell receptors binding and not MHC binding is detected in the assay.

The capacity of peptides to inhibit antigen presentation in in vitro assays, such as those described above, is correlated with the capacity of the peptide to inhibit an immune response in vivo. In vivo activity may be determined in animal models, for example, by administering an antigen known to be restricted to the particular MHC molecule recognized by a target T cell, and the immunosuppressive peptide. T lymphocytes are subsequently removed from the animal and cultured with a dose range of antigen. Inhibition of stimulation is measured by conventional means, e.g., pulsing with tritiated thymidine and comparing to appropriate controls. Certain experimental details will of course be apparent to the skilled artisan. See also, Adorini, et al., Nature 334:623-625 (1988), incorporated herein by reference.

The peptides identified using the assays described above are suitable for treating celiac and other diseases. The peptides can be used in a number of applications. For instance, they may be linked to an appropriate vehicle such as a protein or lipid molecule and administered by intravascular or subcutaneous injection to induce an inflammatory response. They may injected intramuscularly to induce a protective immune response or can be administer orally or parenterally to induce tolerance.

Treatment using peptides which are T cell antagonists offers a number of advantages. For instance, very specific inhibition of an immune response is possible because T cell antagonism is effective against only T cells recognizing the particular antigen involved in the celiac disease. In addition, relatively low doses of peptides are required to inhibit an immune response because inhibition using these peptides is extremely efficient. It is known that as little as about 0.01% to about 0.1% of the MHC glycoproteins on an antigen presenting cell need to be occupied by antigenic peptides to trigger T cell activation (Harding et al., Nature 346:574-576 (1990), which is incorporated herein by reference. If 0.1% to 1% of a population of MHC glycoproteins are occupied by T cell antagonists, a 10-fold excess of antagonist over antigen could exist. Since the resulting complexes competitively inhibit binding, inhibition of T cell activation is achieved.

The peptides of the present invention and pharmaceutical compositions thereof are particularly useful for administration to vertebrates, particularly humans and other mammals, to treat celiac disease. The dose of the immunosuppressive peptides of the invention for treatment of celiac disease will vary according to, e.g., the peptide composition, the manner of administration, the particular disease being treated and its severity, the overall health and condition of the patient, and the judgment of the prescribing physician. Administration should begin at the first sign of symptoms or shortly after diagnosis, and continue at least until symptoms are substantially abated and for a period thereafter. In established cases loading doses followed by maintenance doses may be required.

The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see, Langer, Science 249:1527-1533 (1990), which is incorporated herein by reference.

For the treatment of celiac disease and other food hypersensitivities, oral administration is generally preferred. For instance, the pharmaceutical compositions may be in the form of a yogurt or bacterial broth comprising bacteria that express and secrete the desired protein or peptide. The bacteria colonize the gut and deliver the immunosuppressant peptides or proteins directly to cells involved in the pathology of celiac disease. For oral administration, a pharmaceutically acceptable nontoxic composition is generally formed by incorporating any of the normally employed excipients, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, preferably 25%-75%. For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

The pharmaceutical compositions may also be administered parenterally, i.e., intraarticularly, intravenously, subcutaneously, or intramuscularly. Thus, this invention provides compositions for parenteral administration which comprise a solution of the immunosuppressive peptide molecules dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of immunosuppressive peptides,

which may be combined to form a peptide "cocktail" under certain circumstances for increased efficacy, in the pharmaceutical formulations can vary widely, i.e., from less than about .01%, usually at or at least about 5% to as much as 50 to 75% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 10 mg of peptide. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunosuppressant peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arbitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene

derivatives of these esters. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquified propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquified propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided peptide(s) and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

The compositions containing the immunosuppressive peptides can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient, but generally range from about 1 μg to about 2,000 mg of peptide per day for a 70 kg patient, with dosages of from about 0.5 mg to about 1,000 mg of peptide per day being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances, the relative nontoxic nature and the general lack of immunogeneticity of peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these immunosuppressive compositions.

In prophylactic applications, compositions containing the peptides of the invention are administered to a patient

susceptible to or otherwise at risk of a particular allergic disease to enhance the patient's own immunoregulatory capabilities. Thus, another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of a peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies that react with different antigenic determinants of the antigen. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing an immune response specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 μg to about 5000 μg per 70 kilogram patient, more commonly from about

10 μg to about 500 μg mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

The peptides may also find use as diagnostic reagents. For example, in the case of celiac disease a subcutaneous or intradermal injection of the peptides gives a local inflammatory reaction within 24-48 hours. The concentration required is generally in the range of from about 0.01mg to 5 mg. The peptide can be branched (e.g., multiple linking of the same peptide) or linked to an appropriate protein carrier such as albumin or attached to a lipid molecule (e.g., palmytol)

The following examples are offered by way of illustration, not by limitation.

Examples

Example 1

This example details how T cell antagonist peptides are identified. Briefly, peptide fragments from the appropriate antigen are prepared and screened for the ability to induce T cell activation. Those fragments which induce T cell activation are termed antigenic peptides. Next, analogs of the antigenic peptides are prepared and tested for the ability to bind the MHC molecule and form inhibitory complexes. The exemplified case described in detail below demonstrates the identification of T cell antagonist peptides derived from hemagglutinin of the influenza virus.

1. Identification of Antigenic Peptides

Antigenic peptides capable of inducing T cell proliferation in DR-1 restricted T cell clone (Clone 1) were identified as follows.

A. MHC Purification

EBV-transformed homozygous cell line LG-2, maintained in vitro by culture in RPMI 1640 medium (Flow Laboratories, McLeon, VA), supplemented with 2 mM L-glutamine (GIBCO) and 10% heat-inactivated FCS (Hazleton Biologics Inc., Lenexa, KS) or horse serum (Hazleton Biologics), was used as a source of DR molecules. Cells were lysed at a concentration of 10^8 cells/ml in 50 mM Tris-HCl, pH 8.5, containing 2% Renex, 150 mM NaCl, 5 mM EDTA, and 2 mM PMSF. The lysates were cleared by centrifugation at 10,000 x g for 20 min.

DR molecules were purified using the mAb LB3.1 covalently coupled to protein A-Sepharose CL-4B, as described in O'Sullivan et al. J. Immunol. 145:1799-1808 (1990), which is incorporated herein by reference). Briefly, cell lysates were passed sequentially through the following columns: Sepharose CL-4B, protein A-Sepharose, W6/32-protein A-Sepharose, and LB3.1-protein A-Sepharose washed with 10-column volumes of 10 mM Tris-HCl, pH 8.0, 0.1% Renex (5 ml/h); 2-column volumes of PBS, and 2-column volumes of PBS-1% octylglucoside. The LB3.1 column was eluted with 0.05 M diethylamine in 0.15 M NaCl/1%

octylglucoside (pH 11.5), immediately neutralized with 2 M glycine, pH 2.0, and then concentrated by ultrafiltration.

B. MHC Binding Assays

Purified DR1 molecules (5-10 nM) were incubated with 5 nM ^{125}I-radiolabeled Y(HA 307-319) peptide for 48 hr in PBS containing 5% DMSO 0.05% NP-40 in the presence of a protease inhibitor mixture. The final concentrations of protease inhibitors were: 1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM N-ethylmaleimide, and 200 μM N^{α}-p-tosyl-L-lysine chloromethyl ketone. The DR-peptide complexes were separated from free peptide by gel filtration on Sephadex G50 or TSK columns. In the inhibition assays, peptide inhibitors were added to DR molecules at the same time that radiolabeled peptides were added. Peptide inhibitors were typically tested at concentrations ranging from 120 μg/ml to 1.2 ng/ml. The data were then plotted and the dose yielding 50% inhibition measured.

C. Peptide Synthesis

Peptides were synthesized on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer and radiolabeled as previously described in O'Sullivan et al., supra. Briefly, after removal of the α-amino-tert-butyloxycarbonyl protecting group, the phenylacetamidomethyl resin peptide was coupled with a fourfold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, histidine, asparagine, and glutamine) for 1 hr in dimethylformamide. For arginine, asparagine, glutamine, and histidine residues, the coupling step was repeated in order to obtain a high coupling efficiency. Peptides were cleaved by treatment with hydrogen fluoride in the presence of the appropriate scavengers purified by reversed phase HPLC. The purity of the peptides was substantiated by amino acid sequence and/or composition analysis. They were routinely greater than 95% pure after HPLC.

D. Generation of T Cell Clones and Lines

Peripheral blood mononuclear cells (PBMC) derived from DR1 individuals were cultured (10^5/well) in round-bottomed wells in the presence of antigenic peptides at a final concentration of 10 μg/ml. The media formulation used was RPMI 1640 supplemented with 1 mM Sodium pyruvate, 0.1 mM nonessential amino acids, 0.2 mM L-glutamine, 1000 Units/ml penicillin, 100 mg/ml Streptomycin sulphate, and 5% Human AB serum (RPMI-HS). After five days, rIL-2 (Sandoz, Basel, Switzerland) was added to 10 ng/ml final concentrations, and proliferating cultures were further expanded in rIL-2 and screened for Ag specificity. Clone 1 was obtained by cloning an HA 307-319-specific T cell line by limiting dilution, as described in Krieger et al., J. Immunol. 146:2331-2340 (1991), which is incorporated herein by reference. Clone 1 was kept in culture by periodic (~20 days) restimulation with PHA (1 μg/ml) and irradiated allogenic PBMC. As second cell line which is specific for an antigenic peptide from tetanus toxoid, Line 131.5, was restimulated every two weeks with the TT 830-843 peptide (10 μg/ml) and irradiated DR1 PBMC.

E. T Cell Proliferation and IL-2 Assays

In inhibition assays, mitomycin C-treated or fixed LG-2 APC (4×10^4) were cultured in RPMI-HS in round-bottomed 96-well microtiter plates in the presence of suboptimal Ag dose (20 nM for both HA 307-319 and TT 830-843) and varying concentrations of inhibitor peptide (70 μM to 7nM). After 18 hr, T cells (2×10^4) were added to cultures, and after an additional 24 hr., ^{3}H-Thymidine (1 μCi/well; ICN, Irvine, CA) was added. Cells were harvested 18 hr later onto glass fibre filters, and radioactivity was counted by liquid scintillation on a 1205 Beta plate (LKB, Gaithersburg, MD). In all proliferation assays, cultures were set up in duplicate or triplicate.

For IL-2 assays, 50 μl of the supernatant of Clone 1 T cell cultures (2×10^4 cells/well), stimulated for 24 hr with individual peptides in the presence of LG-2 APC (4×10^4 cells/well), were harvested and then assayed for IL-2 content by adding them to the wells of a flat-bottomed 96-well

microtiter plate containing 7 x 10^3 cells/well of the IL-2-dependent T cell line HT-2 (final supernatant dilution 1:2). After 18 hr., ^{3}H-Thymidine was added to HT-2 cultures, and proliferation was measured after 8 hr.

2. Demonstration of an Antigen-Specific Component in Inhibition of Ag Presentation

We synthesized 68 single amino acid-substituted analogs of the HA 307-319 antigenic peptide and tested them for their capacity to bind purified DR1 molecules. Fifteen of these peptides showed decreased (more than 10-fold) DR1 binding affinities. We tested the remaining 53 analogs that were good binders for their capacity to activate DR1-restricted T cell clone, Clone 1. It was found that 18 of these peptide analogs were incapable of inducing a proliferative response even at concentrations as high as 70 μM (i.e., 10,000-fold excess over the concentration required for 1/2 maximal stimulation by the native antigen).

Having identified nonstimulatory HA 307-319 analogs, we tested their capacity to inhibit proliferation of Clone 1 cells and to compare their efficacy with a panel of other unrelated peptides. Live cells from the DR1-homozygous EBV--transformed B cell line LG-2 were used as APC (Table I). It was found that, as expected from classical inhibition of Ag presentation assay measuring competition for available class II molecules, weak or nonbinding peptides (such as Ova 323-339, λ rep 12-26, and HEL 1-18) were unable to complete with HA 307-319 for Clone 1 activation, while peptides with an affinity for DR1 of $\simeq 5\times10^{-8}$ or higher were capable of inhibition, with IC50% roughly in the 10 to 100 μM range. However, it was also found that, despite DR affinities in the same range of the non-HA-related DR binders, the four HA analogs tested were up to 1000-fold more effective inhibitors of Clone 1 proliferation, with IC50 in the 50-500 nM range.

To test whether this phenomenon was due to an intrinsically high inhibitory capacity of HA-related sequence per se, or rather was reflective of an antigen-specific component in inhibition of antigen presentation, the following

experiments were performed: 42 analogs of another DR1-restricted determinant (TT 830-843) previously tested for their DR1 binding capacity, were tested for their capacity to activate the DR1-restricted T cell line 131.5. In analogy to the strategy employed for HA 307-319, analogs showing no decrease in DR1 binding, and also nonstimulatory up to the 100 µg/ml level, were selected for further analysis (data not shown). A selected panel of four HA analogs and four TT analogs were then tested for their capacity to inhibit antigen presentation using live LG-2 cells and either HA- or TT - specific T cells (Table II). The results obtained clearly demonstrate that the previously detected highly efficient inhibition of antigen presentation is antigen-specific, inasmuch as only HA analogs and not TT analogs are capable of inhibiting the HA clone, and conversely that the TT are much more effective inhibitors of TT-specific DR1-restricted T cells than the HA analogs. Similar results were obtained using either live or fixed APCs (data not shown), thus ruling out the possibility that recycling of class II molecules or induction of a negative signal from the APC to the T cells were responsible for the phenomenon observed.

TABLE I

HA Analogs Are Highly Efficient Inhibitors of an HA-Specific T Cell Clone

Peptide	Sequence	Inhibitory Activity on Clone 1 (HA-specific) DR1 Binding	50% Dose (μM)
HA 307-313	P K Y V K Q N T L K L A T	1.00	ND
601.20	- - - - - - Q - - - - - -	0.92	0.06
601.21	- - - - - - T - - - - - -	2.4	0.09
601.07	- S - - - - - - - - - - -	2.10	0.19
713.07	- - - - V - - - - - - - -	0.13	0.32
Nase 101-120	E A L V R Q G L A K V A Y V Y K P N N T	2.30	14.2
MBP 74-98	G R T Q D E N P V V H F F K N I V T P R T P P P	0.16	23.3
Wiley's Poly	A A Y A A A A A A K A A A	1.8	38.0
TT 830-843	Q Y I K A N S K F I G I T E	0.12	56.5
Mat 19-31	P L K A E I A Q R L E D V	0.5	162.0
Nase 81-100	R T D K Y G R G L A Y I Y A D G K M V N	0.16	>300
HEL 1-18	K V F G R C E L A A A M K R H G L D	0.05	>300
Ova 323-339	I S Q A V H A A H A E I N E A G R	-	>300
Lambda rep 12-26	L E D A R R L K A I Y E K K K	-	>300

TABLE II

The Phenomenon of Antigenic Preference

Peptide	Sequence	Relative Binding to Purified DR1	50% μM Dose [1] for Inhibition of Clone 1 (HA-specific)	50% μM Dose [1] for Inhibition of A31.5 (Tet tox-specific
HA 307-319	P K Y V K Q N T L K L A T	1.00	ND	ND
601.07	- S - - - - - - - - - - -	2.10	0.6	119.2
713.07	- - - - V - - - - - - - -	0.13	1.1	106.4
601.20	- - - - - - Q - - - - - -	0.92	0.5	109.2
601.21	- - - - - - - T - - - - -	2.40	0.4	103.6
TT 830-843	Q Y I K A N S K F I G I T E	0.17	ND	ND
650.11	- - - S - - - - - - - - -	0.14	115	5.8
650.17	- - - - - - - A - - - - -	0.11	>200	18.9
650.18	- - - - - - - L - - - - -	0.03	>200	8.8
650.25	- - - - - - - - - R - - -	0.44	>200	19.6

[1] Average of at least two independent experiments.

3. Lack of Differential Inhibitory Capacity of Antigen Analogs at the Level of DR-antigen Interaction

To provide a molecular explanation for the phenomenon observed, we considered the possibility that the TT and HA peptides may bind somewhat different subsites on the DR1 molecule, thus rendering inhibition by the homologous antigen analogs more effective.

In the series of experiments shown in Table III, HA 307-319 and TT 830-843 peptides were radiolabeled and then assayed for their capacity to be inhibited by either HA or TT analogs. No significant difference was found between the relative capacity of either HA or TT peptides to be inhibited by either TT or HA analogs, thus formally ruling out the possibility that the observed antigen-specific component of inhibition of antigen presentation might be reflective of differential inhibitory capacity at the level of the DR1 molecule.

TABLE III

Inhibitory Capacity of Ag Analogs

at the Level of DR1 Binding

Peptide	Sequence	50% nM Doses [1] for Inhibition of DR1 Binding	
		I^{125} HA Peptide	I^{125} TT Peptide
HA 307-319	P K Y V K Q N T L K L A T	4.9±1.9	3.2±1.0
601.07	- S - - - - - - - - - - -	1.4±0.7	0.6±0.1
713.07	- - - - V - - - - - - - -	2.0±0.9	1.1±0.5
601.20	- - - - - - Q - - - - - -	3.6±2.4	1.4±0.2
601.21	- - - - - - T - - - - - -	4.0±1.9	1.7±0.4
TT 830-843	Q Y I K A N S K F I G I T E	54±4	30±11
650.011	- - - S - - - - - - - - -	51±26	27±5
650.12	- - - I - - - - - - - - -	19±13	7.4±2.8
650.17	- - - - - - - S - - - - -	63±10	42±12
650.18	- - - - - - - L - - - - -	280±185	200±71
650.25	- - - - - - - R - - - - -	30±6	40±21

[1] Averages of at least three independent experiments.

4. Lack of Direct Inhibitory Capacity of the Antigen Analogs at the T Cell Level

We next hypothesized that the effect might be somehow mediated by direct interaction of the antigen analogs with either the T cell receptor or other T cell molecules expressed on the T cell membrane (such as, for example, DR molecules). To rule out this possibility, we performed the experiment shown in Figure 1. A dose range of the HA analog 601.20 (having the sequence PKYVKQQTLKLAT) was incubated for two hours with either T cells or with APCs which were simultaneously incubated with the antigenic peptide. At the end of two hours of incubation, the two cell populations were washed and recombined. Thymidine incorporation was measured three days later, as usual. The results obtained indicate that pulsing Clone 1 T cells with the HA analogs does not inhibit the proliferative response, while incubation of the APC population with the same HA analog induces a profound dose-dependent inhibition of T cell proliferation.

5. Detection of T Cell Activation

A recent report suggests that antigen analogs may induce a partial activation of T cells, as evidenced by cytokine production in the absence of the induction of proliferation (Evavold et al., Science 25:1308-130 (1991), which is incorporated herein by reference). To investigate whether a similar mechanism might be operative in our experimental system, we analyzed the patterns of lymphokine production by Clone 1 T cells in response to either Ag/DR1 complexes or Ag analog/DR complexes. Since preliminary experiments showed that these T cells produced IL-2 and no IL-4 (data not shown), only the IL-2 response was studied. To measure IL-2 production, culture supernatants were tested for their capability to sustain growth of the IL-2 dependent HT-2 cell line, as described above. It was found (Figure 3) that MHC-antigen peptide complexes induced production of readily detectable amounts of IL-2 down to 7-70 nM antigen doses. By contrast, the HA analog 601.20 induced no detectable IL-2 responses, even at concentrations as high as 70 μM.

We also analyzed whether HA analog/DR1 complexes could induce some of the early biochemical events associated with T cell receptor-mediated activation. To do this, we measured the capacity of HA 307-319 or of the analog peptide 601.20 to stimulate increases in inositol phosphate (IP) production. Clone 1 T cells were labeled with 3H-inositol, washed, and suspended in media containing 20 mM LiCl. The T cells were co-cultured with LG-2 APC that had been prepulsed with or without various concentrations of HA 307-319 or 601.20 as indicated. After 1 hr, the cultures were organic extracted, and the aqueous layer was analyzed for total inositol phosphates. The data were expressed as the amount of radioactivity recovered from the IP fraction divided by the total amount of radioactivity incorporated by the T cells (X100). The values obtained were the average and standard error of duplicate samples. The results (Figure 4) reveal a dose-dependent increase in IP formation when T cells were stimulated with APC prepulsed with antigenic peptide HA 307-319. The response was detected using doses of HA 307-319 as low as 70 nM, whereas, in contrast, analog peptide doses up to 70 μM did not induce any detectable increase in IP formation. Similar results were obtained with Ca^{++} flux experiments (data not shown).

6. Lack of Demonstrable Negative Signaling

Another possibility examined was that the antigen analogs would act neither as MHC competitors nor interact with the T cell receptor, but rather that the antigen analog could, once bound to DR molecules, induce a negative response, such as tolerance, T cell anergy, or apoptosis. Schwartz, Science 248:1349-1356 (1990), which is incorporated herein by reference. To test this, two different kinds of experiments were performed. In the first experiment, we followed a protocol originally described by Lamb et al, J. Exp. Med. 157:1434-1447 (1983), which is incorporated herein by reference. We incubated Clone 1 cells for 20 hr in absence of APC but in presence of high doses (100 μg/ml) of either the antigenic HA 307-319 peptide or its nonstimulatory analog,

601.20. HA 307-319 preincubation resulted in induction of T cell unresponsiveness to a subsequent challenge with the HA 307-319 peptide presented by the LG2 APCs (Figure 2). By contrast, preincubation with the HA analog 601.20 did not result in any reduction of the response to the subsequent antigen/APC challenge. These results suggest that the mechanism for the inhibition detected in the case of 601.20 must be other than induction of T cell tolerance. Similarly, no inhibitory effect was demonstrated when Clone 1 T cells were stimulated with PHA in the presence of a dose range (160 to 0.016 μg/ml) of the 601.20 peptide (data not shown). Taken together, these results argue against a role for T cell tolerance or negative signaling in the inhibitory activity detected for antigen analogs.

7. Dissociation of MHC Competition from Antagonism of the T Cell Receptor

Having excluded the three most obvious possibilities to explain the observed phenomenon, the only other plausible possibility is the inhibiting peptides or T cell antagonists.

A complicating feature of the inhibition of antigen presentation assays performed thus far, in which antigen and competitor are added simultaneously to the APCs, is that antigen analogs can act simultaneously as: 1) MHC blockers, competing for the peptide binding site of DR1 molecules, and 2) as illustrated above, could also act by generating analog-MHC complexes capable of competing with antigen-MHC complexes for binding to the T cell receptor.

To exclude any influence of MHC competition and discrimination between these two mechanisms, a "prepulse" assay, in which LG-2 cells are pulsed for two hours at 37° with suboptimal doses of the HA antigen, was defined. After removing unbound antigen by washing the cells, the HA analogs are added to the APC cultures. It should be noted that under these experimental conditions, only a small number of DR molecules will be occupied by the HA analog. As discussed above, suboptimal T cell responses are induced by as little as 50 to 300 complexes, corresponding to as little as 0.01-0.1% of

total class II, Schwartz, supra. Since peptide-MHC complexes are, in general, very long-lived (in the case of HA 307-319/DR1, the $t^{1/2}$ at 30°C is in the order of a few days, the addition of HA analogs subsequent to the antigen pulse should not result in competition at the DR1 level.

The "prepulse" assays were performed as follows. Paraformaldehyde-treated LG-2 APC (2.7×10^5/ml) were incubated with a suboptimal concentration of HA 307-319 (35 nM) for two hr at 37°C, then washed and plated at 4×10^4/well in the presence of varying concentrations of inhibitor peptides (100 μM-5 nM) and 2×10^4 T cells. After 48 hr, cultures were pulsed with ^{3}H-Thymidine, and proliferation was measured as described above.

The results obtained (Figure 5A) verify that peptides unrelated to HA act as inhibitors of antigen presentation only in the classical MHC competition assay, but are, as expected, devoid of any activity in the prepulse assay.

The capacity of antigen analogs to inhibit in prepulse assays in an antigen-specific manner was examined next. It was found that in prepulse assays (Figures 5B and 5C), HA analogs act as potent inhibitors of the HA-specific Clone 1, but have no effect on the proliferation of the TT 830-843-specific T cell line 131.5. Conversely, it was found that TT analogs are potent inhibitors, in prepulse assays, of the TT-specific T cells, but had no effect on the inhibition of the HA-specific Clone 1 T cells.

Example 2

This example shows which class II restriction elements are responsible for antigen presentation in celiac disease. In addition, through the use of a panel of synthetic peptides spanning through the entire alpha gliadin sequence, epitopes which are recognized by alpha gliadin-specific T cells are identified.

Materials and Methods

Donors

The study population consisted of three normal individuals and two patients with celiac disease (CD).

Normals. Donor 1 (JA) was a 40-year-old Caucasian male with an HLA-DR3,7 haplotype; Donor 2 (JR) was a 31-year-old Caucasian male with an HLA-DR3,7 haplotype; Donor 3 (MC) was a 43-year-old Caucasian male with an HLA-DR3,4 haplotype.

Patients. Patient 1 (RCL) was a 74-year-old Caucasian male with a long history of CD, in clinical status of remission at the time of the study, with an HLA-DR5,7 haplotype. Patient 2 (AA) was a 65-year-old female with active disease at the time of the study, bearing an HLA-DR7,11 haplotype. Both patients are predicted to express DQ2.3 heterodimer that has been shown to be strongly associated with CD. The healthy controls selected for this study are also predicted to be all DQ2.3-positive, on the basis of their DR and DQ haplotypes.

Antigens

Whole alpha gliadin derived from unbleached flour (bread wheat variety Scout 66) was purified with the method previously described by Bernardin et al. J. Biol. Chem. 242:445 (1967), which is incorporated herein by reference. Twenty-five synthetic peptides 20 amino acids in length, overlapping by 10 amino acids, spanning the entire alpha gliadin sequence and 15 truncated peptides for further fine specificity study were generated, following the procedures previously described by O'Sullivan et al., supra. The rabies peptide 285-299 DALESIMTTKSVSFR was also synthesized and used as a control antigen (see below). Irradiated PBMC from a DR3,7 healthy donor were used as stimulators for the DQ2.7-specific alloreactive T cell clone (JS 87).

Proliferation assay

T cell lines and clones were washed extensively and incubated (5×10^4) in 200 μl of complete medium plates with in flat-bottomed wells (Falcon Labware, Oxnard, CA) for 72 hr in duplicate with 5×10^4 irradiated autologous or DR3 (Mat), DR5 (Sweig), or DR7 (Pitout) homozygous EBV-transformed B cell lines or murine DR-transfected fibroblasts (provided by R.

Karr, Monsanto Co., St. Louis, MO), in the presence of different amounts of antigen. Wells containing APCs and T cells in the absence of antigen were used as controls. For fine specificity experiments, APCs were fixed for 30 sec with glutaraldehyde at a final concentration of 0.025% (Sigma Chemical Co., St. Louis, MO), quenched by FCS, washed extensively, and plated using the same protocol as for irradiated APCS. Eighteen hours before harvesting cultures, 1 μi of ^3H thymidine (ICN, Irvine, CA) was added to each well. Radioactivity retained after harvesting (1295-001 cell harvester, LKB, Gaithersburg, MD) in the filters was measured in a liquid scintillation counter (1205 beta plate, LKB, Gaithersburg, MD). Results were expressed in mean cpm+ SEM.

Blocking experiments

MHC isotype restriction was determined by inhibition of proliferative responses by a panel of purified monoclonal antibodies (mabs) (anti-DR antibody LB3.1, anti-DQ2 antibody 11B.5, and anti-DP antibody B27.21). mAbs were purified by Protein A affinity chromatography and added at 10 μg/ml to duplicate wells containing autologous or homozygous, heterologous irradiated EBV-B cell lines. After 1 hr incubation at 37°C in 5% CO_2, T cells from the gliadin-specific T cell lines were added together with antigen. Two previously characterized clones, DR- and DQ-restricted, respectively, were used as controls. The DR-restricted clone, specific for a Rabies viral glycoprotein peptide in the context of DR7 class H molecules, was kindly donated by E. Celis (Cytel Corp., San Diego, CA). The anti-DQ2.7 alloreactive T cell clone was kindly donated by H. Serra (Cytel Corp., San Diego, CA). The cultures were incubated for 72 hrs, then pulsed with 1 μCi of ^3H thymidine, and incorporation was measured by liquid scintillation counter, as described below.

Generation of gliadin-specific T cell lines and clones

Antigen-specific T cell lines and clones were derived from peripheral blood mononuclear cells (PBMCs) from celiac disease patients and normal donors. PBL were plated at 2 x 10^5

per well in 96 well flat-bottomed plates (Falcon Labware, Oxnard, CA) in the presence of whole alpha gliadin at a concentration of 100 μg/ml. Twenty U/ml r-IL2 (Sandoz, Basel, Switzerland) was added after 5 days. After an additional 5-6 days, growing cultures were expanded with r-IL2 and maintained on a 15-day cycle of re-stimulation with antigen plus autologous irradiated (irr.) PBMCs as antigen-presenting cells (APCs). Specific lines were cloned by limiting dilution of 0.5 and 1 cell per well with 1 μg/ml PHA (Wellcome, Dartford, UK), r-IL2 and allogeneic irr.-PBMCs, as described by Barnaba et al., Nature 345:258 (1990).

Results

Generation of gliadin-specific T cell lines

Gliadin-specific T cell lines were generated by repeated *in vitro* stimulation with intact purified alpha gliadin of peripheral blood mononuclear cells (PBMC) derived from two different celiac disease patients. Three normal healthy individuals were also studied. Both patients were DR5,7 heterozygous and would therefore be predicted to express the DQ2.3 heterodimer which has been shown to be strongly associated with celiac disease. At the time of bleeding, the patient (RCL) was in remission, while patient AA had active disease. The three healthy controls selected for this study were afl also predicted to express the DQ2.3 heterodimer, on the basis of their DR and DQ haplotype [DR3,7 (JA); DR3,7 (JR); and DR3,4 (MC)].

After ficoll isolation, replicate cultures were set up for each individual and stimulated with 100 μg/ml of purified alpha gliadin, using irradiated autologous PBMC as APCS. Lines were then restimulated every 15 days as described in Materials and Methods. Before every round of stimulation, specific antigen proliferation responses were assessed. The lines that showed high background proliferation (more than five times the proliferation level of unstimulated controls) were eliminated, while lines showing no detectable specific response, but with low background, were restimulated further. After the second round of stimulation, gliadin-specific lines

were obtained from one of the normal donors (JR) and one of the two celiac patients (RCL). No specific lines were obtained after as many as four restimulations from the remaining two normal donors and the other celiac patient. The alpha gliadin-specific proliferative responses of the two lines obtained are shown in Figure 6. In order to avoid, as much as possible, selecting *in vitro* for particular specificities, the two lines were immediately (after the second round of stimulation) cloned. At the same time, the lines were also expanded further and characterized for their class II restriction.

MHC restriction of gliadin-specific T cell lines

To define the MHC restriction of the two lines obtained, both inhibition experiments with specific mabs, as well as genetic mapping experiments, were performed. The inhibition of proliferative responses to alpha gliadin of the two T cell lines by mAbs specific for different human MHC class 11 isotypes was analyzed first (Figure 7). The anti-DR monoclonal LB3.1 drastically inhibited the responses of both JR and RCL lines, as well as the control DR-restricted, rabies-specific clone, C25. Nonspecific effects were ruled out by the lack of inhibition of the same mAb on the DQ2.7-restricted alloreactive T cell clone, JS87.

As anticipated, the DQ-specific mAb 11.B5 was effective in inhibiting the proliferation of the DQ2.7-restricted alloreactive clone JS87, but was ineffective against the control DR-restricted clone C25, and no inhibition was detected in the case of either JR or RCL gliadin-specific line. Finally, no inhibition was detected for any of the lines and clones tested in the case of the anti-DP antibody B27.21. Taken together, these data indicate that the gliadin-specific T cell lines obtained are mostly or totally DR-restricted.

The MHC restriction of the two gliadin-specific lines was further mapped using a panel of either autologous (DR3,7 and DR5,7, respectively), or homozygous (DR3, DR5, or DR7) EBV-transformed B cell lines (Figure 8). In the case of the JR line, good presentation was observed with the autologous DR3,7

EBV line and by the homozygous DR7 EBV line (Figure 3A). By contrast, no response was detected in the case of DR5 and DR3 EBV lines.

On the basis of these data and the mAb inhibition data presented above, it was concluded that either DR7 or DR53 molecules, the product of the linked DRβ2 locus, could be acting as a restriction element for the JR line. The antigen-presenting capacity of murine fibroblasts transfected with either DR7 (or the product of the linked DR DR53 molecules was therefore analyzed to clarify this issue. The results, also shown in Figure 8A, revealed that the JR line was DR53-restricted. In the case of the RCL line, following an analogous strategy, DR7 was also mapped as the restriction element (Figure 8B). In this case, however, both DR53 and DR7 fibroblasts appeared to be capable of presenting antigen. Results obtained with clones derived from this line suggest that this result was due to the presence in the same T cell line of two different T cell populations, one restricted by DR7 and one by DR53. It should also be noted that the absence of any proliferation by both JR and RCL lines, when the DR3 homozygous EBV lines (expressing DQ2.3 molecules) were used as APCS, further confirmed the lack of DQ2.3-restricted T cells in the alpha gliadin-specific lines.

Fine specificity of gliadin-specific T cell clones

The specific lines described above were cloned by limiting dilution in the presence of PHA, rIL2 and irradiated allogeneic feeder cells. In total, seven alpha gliadin-specific clones were generated from the JR line (normal donor), and four alpha gliadin-specific clones were generated from the RCL line (celiac patient). These clones were tested against a panel of 25 synthetic peptides spanning the entire alpha gliadin sequence, 20 amino acids in length and overlapping by 10 amino acids, using irradiated homozygous DR7 EBV-B cells as APC. The purpose of this experiment was to determine which residues of the alpha gliadin sequences were recognized by the different clones. It was found that all the clones derived from the normal donor recognized alpha gliadin 21-40 peptide.

Representative data for two of them are shown in Table IV. Following a similar strategy, the determinant(s) recognized by clones derived from the RCL line was also investigated. In this case, it was found that all the clones derived from the celiac patient proliferated in response to the N-terminal peptide alpha gliadin 1-20 (Table IV).

Differential antigen processing and presenting capacities of EBV cell lines and DR-transfected murine fibroblasts

In the course of the MHC restriction analysis of the patient-derived, alpha gliadin-specific T cell clones, comparisons were made between DR-transfected murine fibroblasts and EBV cell lines in their capacity to present alpha gliadin. Surprisingly, when intact alpha gliadin was used as an antigen, it was found that much greater stimulation of the T cell clones were obtained with DR-transfected fibroblasts than homozygous EBV cell lines. Typical dose response analysis is shown in Figures 9A and 9B. In contrast, and consistent with previous studies with transfected fibroblasts, when a PPD-specific response was studied, EBV lines were shown to be more efficient APCs than DR-transfected fibroblasts (Figure 9C).

These data suggested the possibility that murine fibroblasts had a greater capacity to process the alpha gliadin than the human EBV cell lines. To address this possibility, these two cell types were compared in their antigen-presenting capacity with the synthetic alpha gliadin peptide 1-20 (Figure 10A). Although the differences were somewhat less marked, it is clear that even with the synthetic peptide, the transfected fibroblasts were still considerably more efficient APCs than the EBV cell line. These results suggested two possibilities: (1) that the difference between the EBV cells and the fibroblasts was still due to processing, despite the fact that typically, peptides of this nature do not require processing, or (2) that other factors, such as unique co-stimulatory factors or accessory molecules, might differ between these two cells and result in the differential APC capacity. Against the second hypothesis was the finding that the difference appeared to be antigen-specific, as suggested by the converse

antigen-presenting capabilities expressed by these two cell lines when a different antigen was used, viz PPD.

To examine the former possibility, i.e., that a processing requirement was necessary for the presentation of the alpha gliadin 1-20 peptide, a series of N- and C-terminal truncations of this peptide were analyzed to determine the minimal antigenic epitope within this 20-residue peptide. The capacity of the truncated peptides to stimulate a response when irradiated EBV cells were used as APCs is shown in Table V. C-terminal truncations led to a gradual increase in the capacity to stimulate a T cell response up to the point when L8 was removed. At the N-terminal end, removal of the first residue eliminated activity. Therefore, alpha gliadin peptide 1-8 was defined as the minimal epitope. When this peptide was used to study the T cell responses induced by irradiated transfected fibroblasts and EBV cells, it was found that fibroblasts and the EBV cell line had similar antigen-presenting capacity (Figure 10A), thus supporting the concept that the difference between these two cell types lay in their differing capacity to process alpha gliadin and the alpha gliadin 1-20 peptide. In further support of this conclusion was the finding that aldehyde-fixed EBV cells were also capable of presenting the alpha gliadin 1-8 peptide efficiently (Figure 10B)

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A composition comprising a peptide capable of inhibiting celiac disease, wherein the peptide binds an MHC molecule on an antigen presenting cell associated with celiac disease and thereby inhibits activation of a T cell associated with celiac disease.

2. The composition of claim 1, wherein the peptide inhibits binding of an antigenic peptide to the MHC molecule.

3. The composition of claim 1, wherein the peptide is a T cell antagonist peptide and binds to the MHC molecule to form an inhibitory complex.

4. A composition of claim 1, wherein the peptide consists of between about 5 and about 25 amino acids.

5. A composition of claim 1, wherein the peptide comprises an amino acid mimetic.

6. A composition of claim 1, wherein the peptide comprises an peptide bond mimetic.

7. A composition of claim 1, wherein the peptide comprises a D-amino acid.

8. A composition of claim 1, wherein the antigen presenting cell expresses DR7 or DR53 MHC glycoproteins.

9. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide capable of inhibiting celiac disease, wherein the peptide binds an MHC molecule on an antigen presenting cell associated with celiac disease and thereby inhibits activation of a T cell associated with celiac disease.

10. The composition of claim 9, wherein the peptide

inhibits binding of an antigenic peptide to the MHC molecule.

11. The composition of claim 9, wherein the peptide is a T cell antagonist peptide and binds to the MHC molecule to form an inhibitory complex.

12. A method of treating celiac disease in a patient comprising administering to the patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide capable of inhibiting celiac disease, wherein the peptide binds an MHC molecule on an antigen presenting cell associated with celiac disease and thereby inhibits activation of a T cell associated with celiac disease.

13. The composition of claim 12, wherein the peptide inhibits binding of an antigenic peptide to the MHC molecule.

14. The composition of claim 12, wherein the peptide is a T cell antagonist peptide and binds to the MHC molecule to form an inhibitory complex.

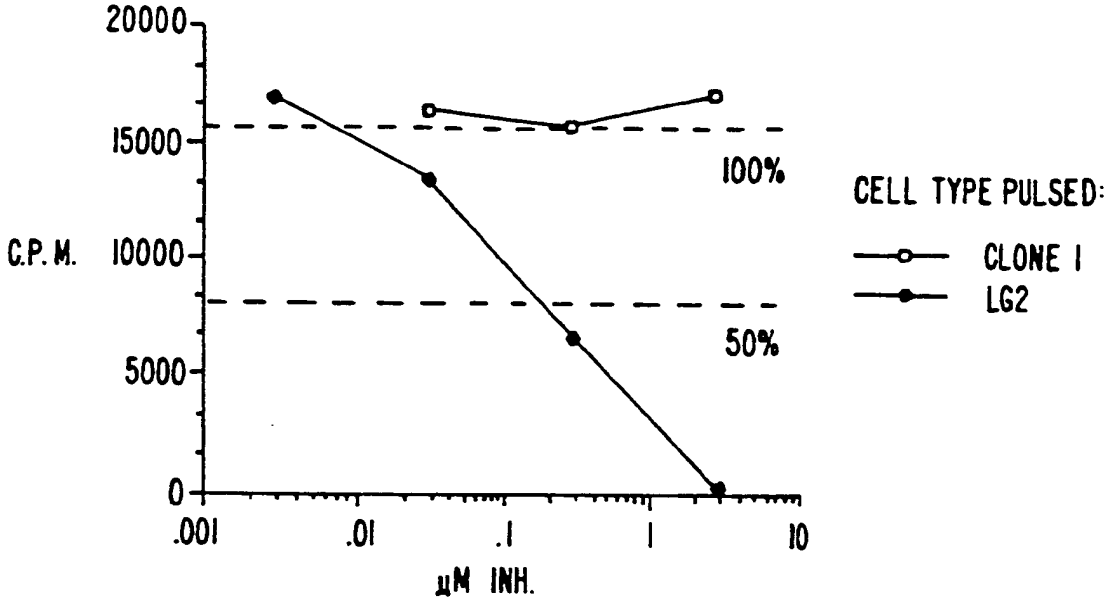

FIG. 1.

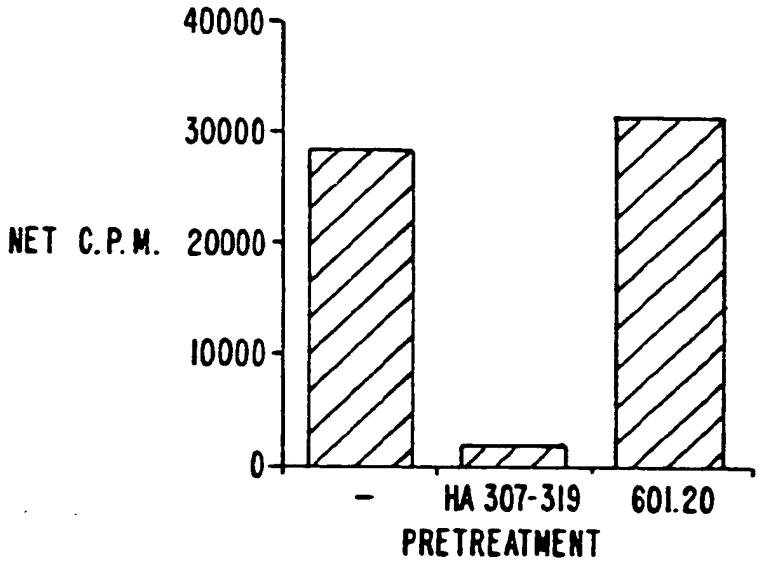

FIG. 2.

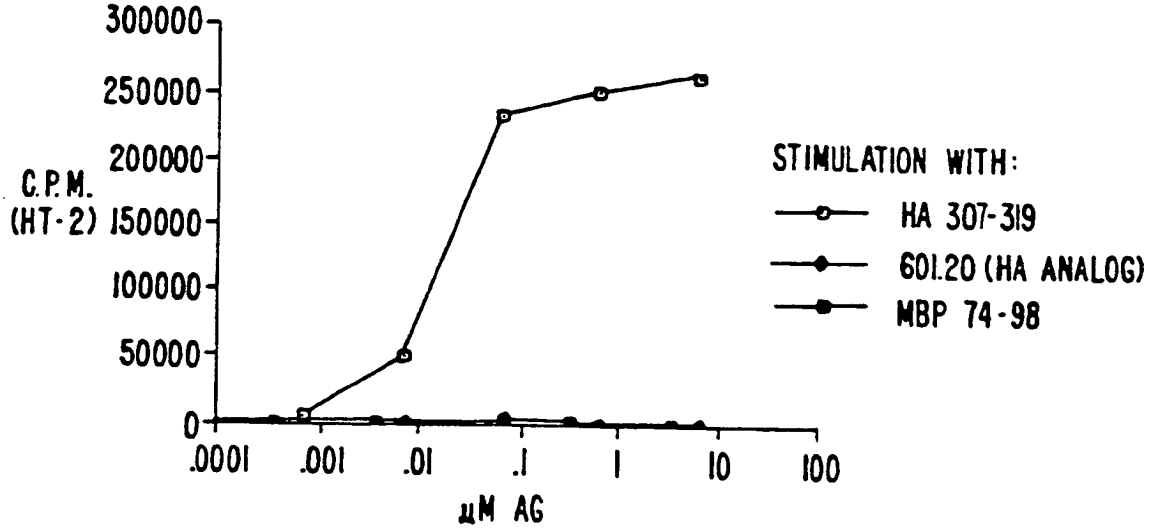

FIG. 3.

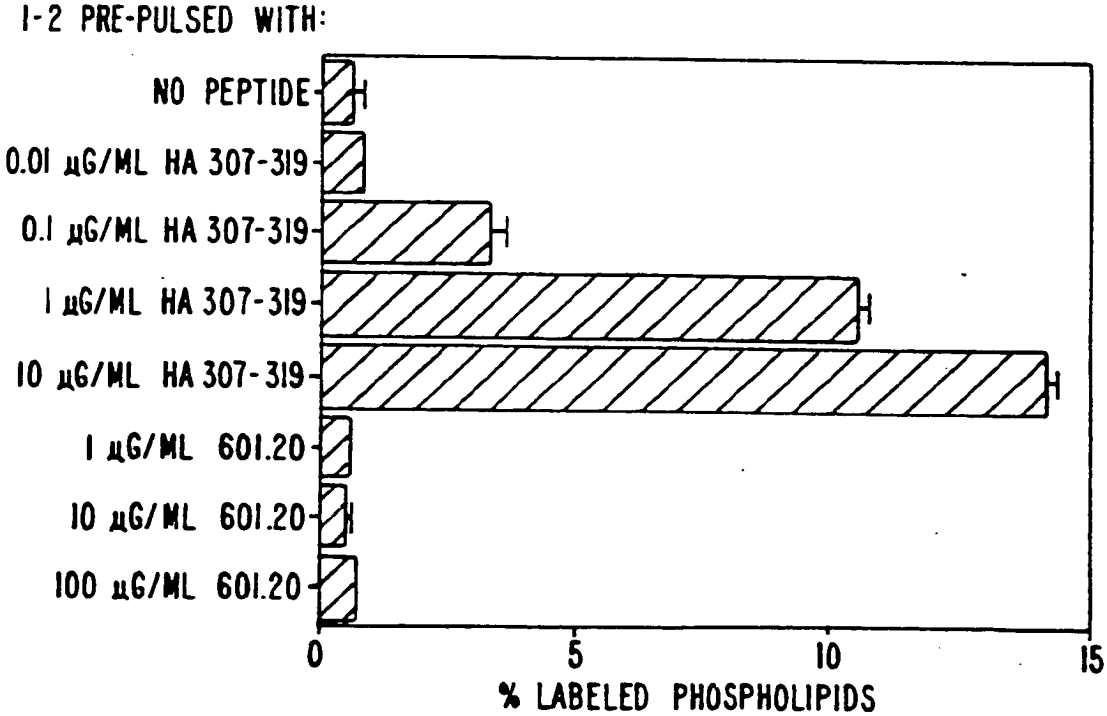

FIG. 4.

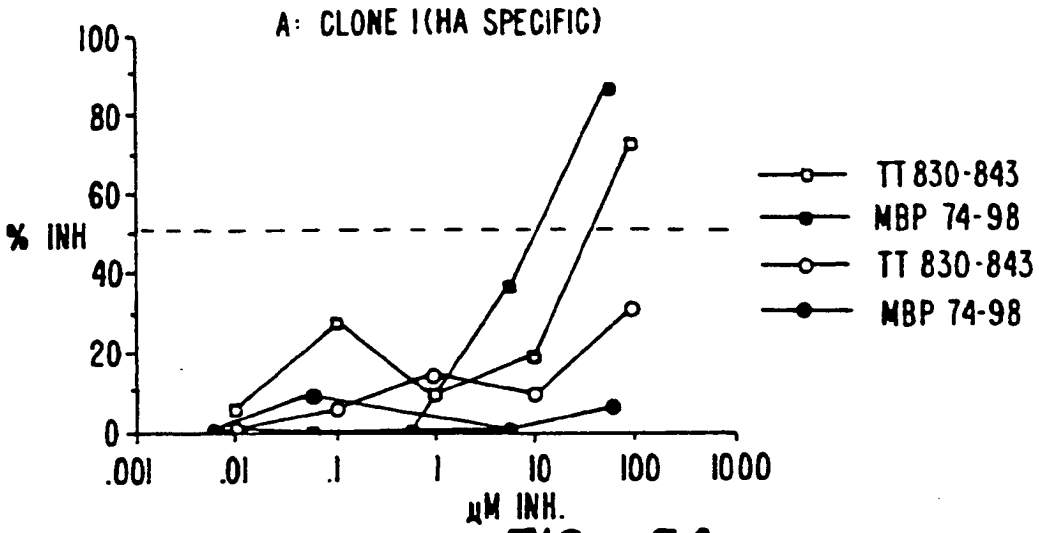

FIG. 5A.

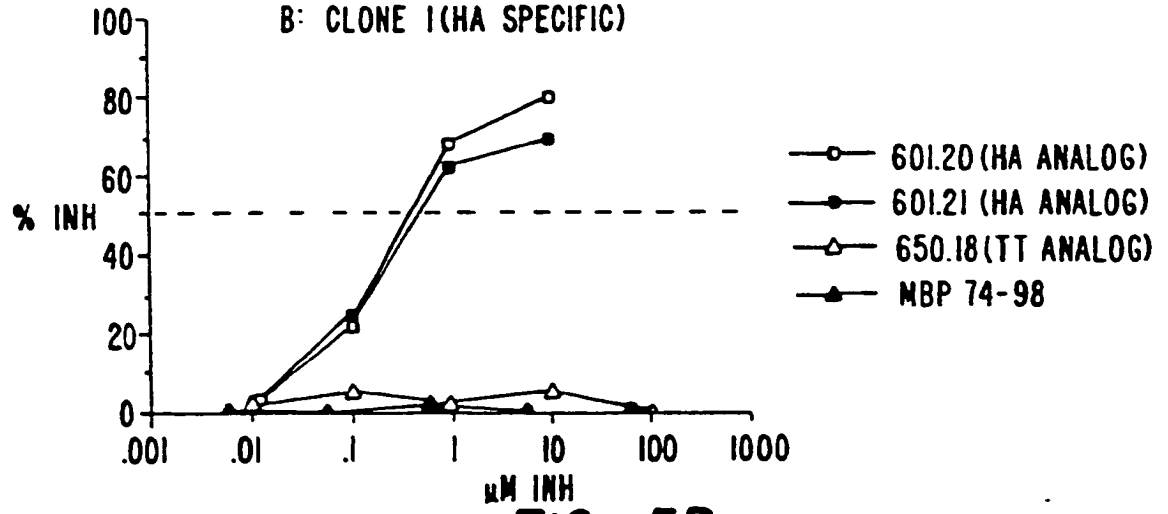

FIG. 5B.

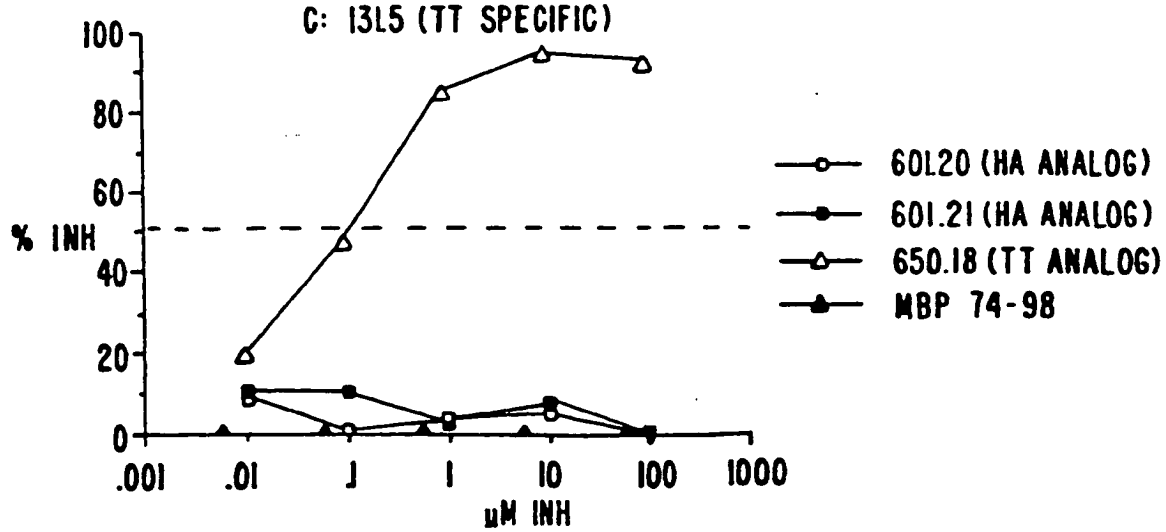

FIG. 5C.

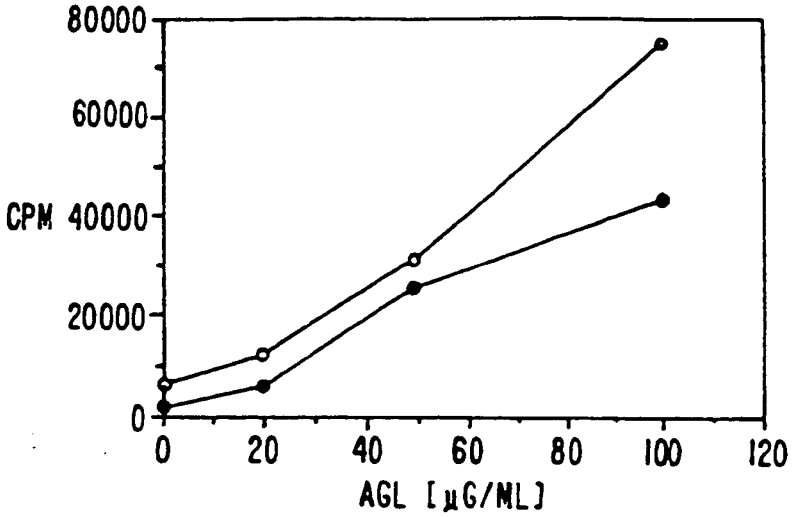

FIG. 6.

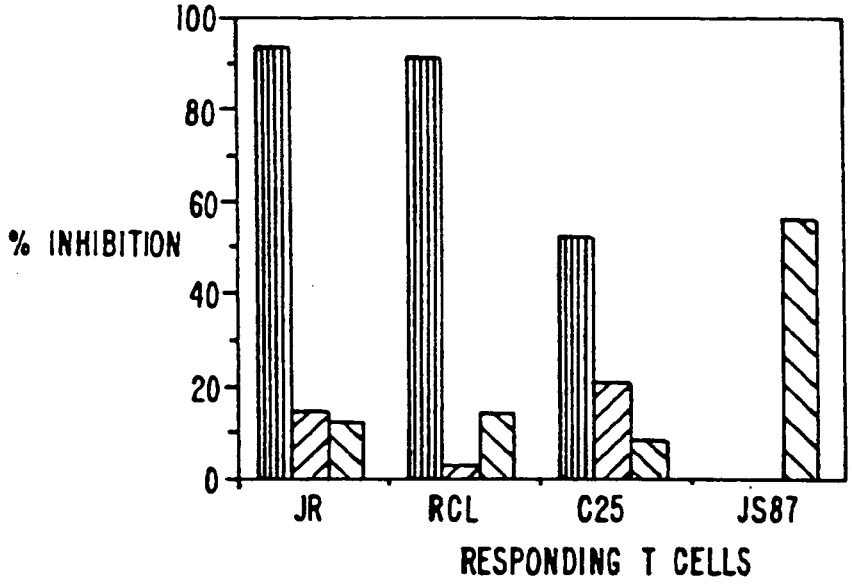

FIG. 7.

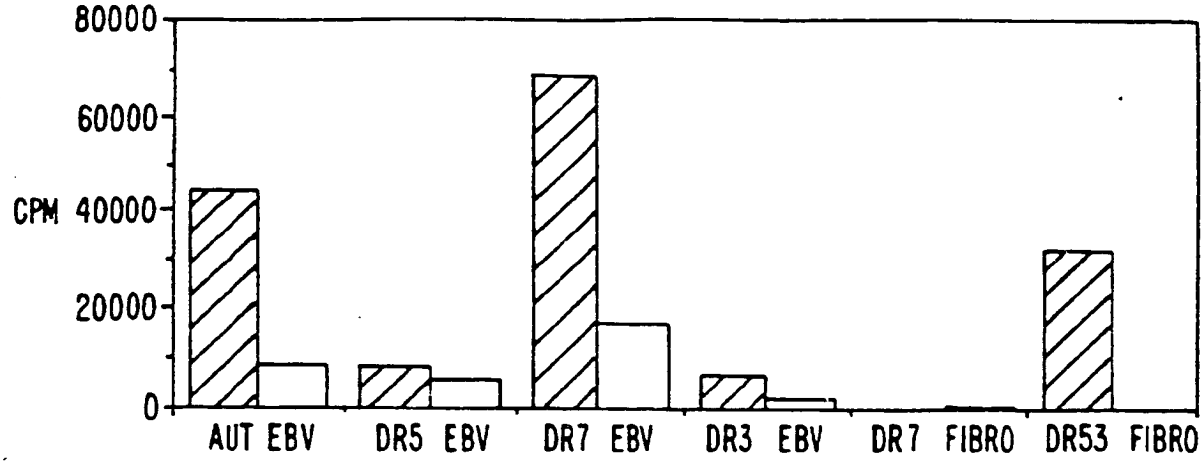

FIG. 8A.

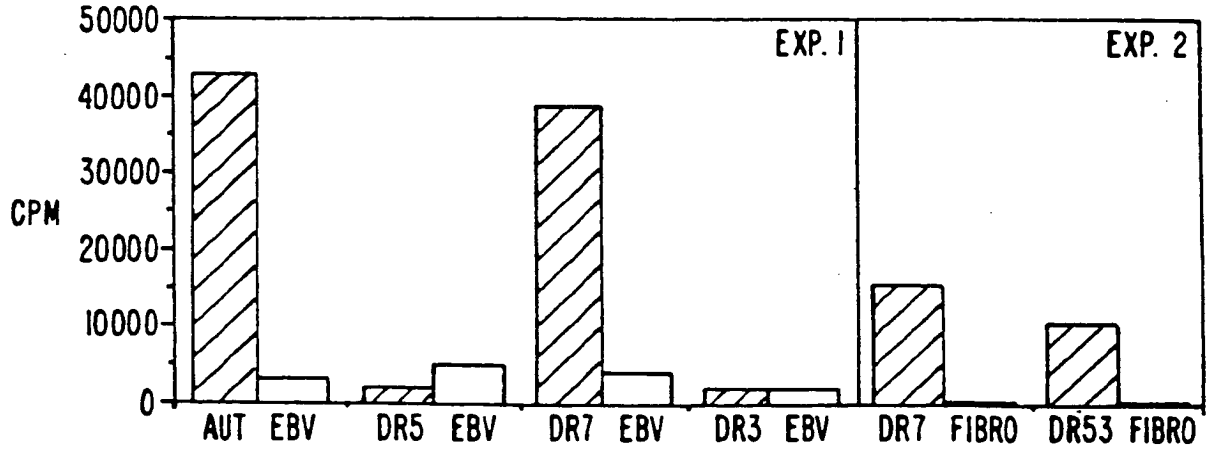

FIG. 8B.

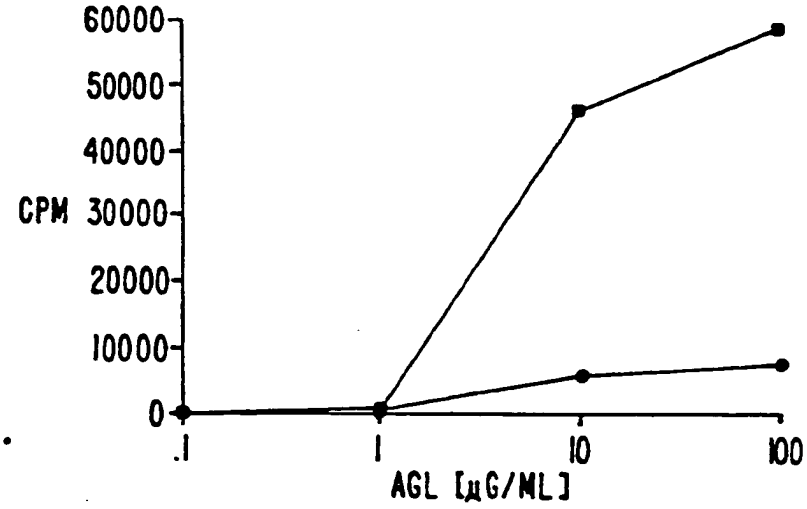

FIG. 9A.

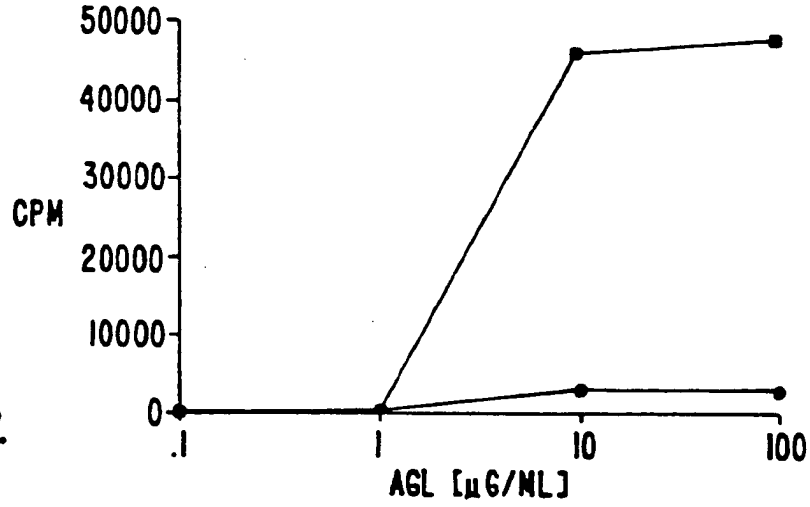

FIG. 9B.

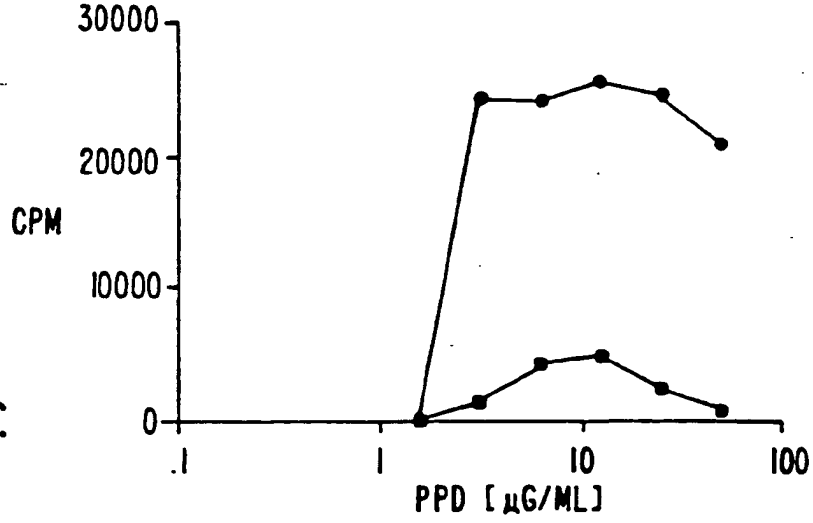

FIG. 9C.

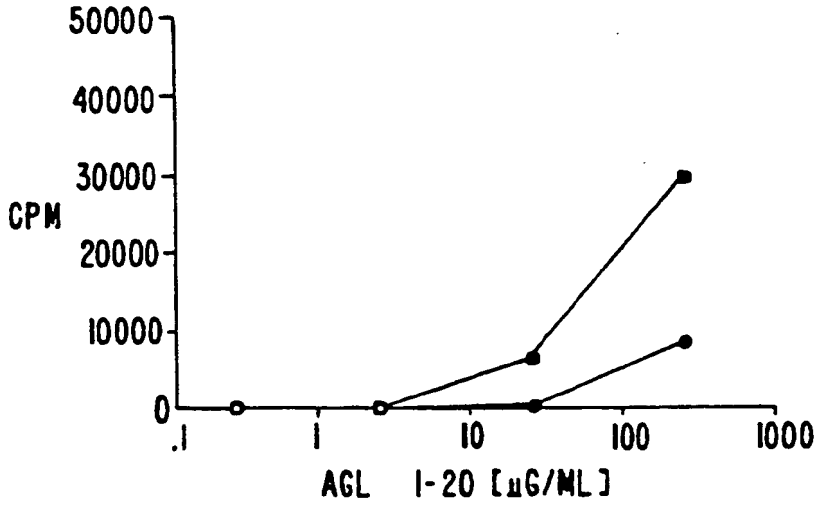

FIG. 10A.

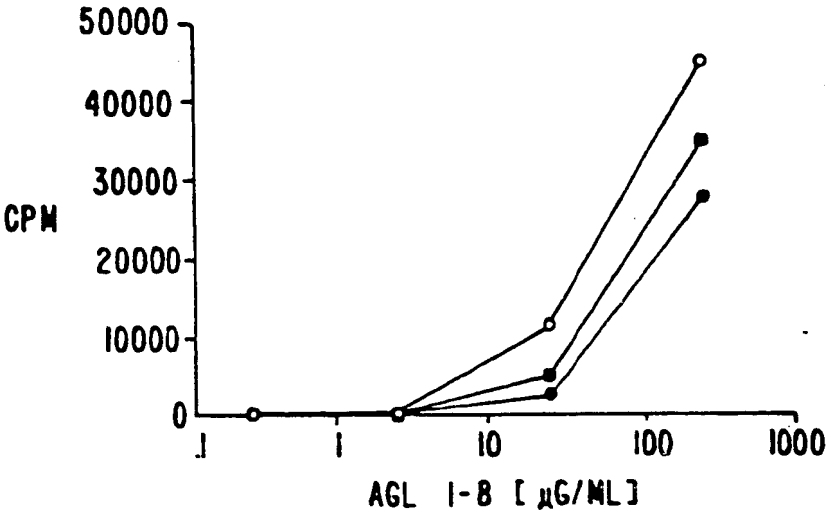

FIG. 10B.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05632

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 7/00, 7/02; A61K 37/02, 39/00.
US CL :514/2. 8; 424/88; 530/300, 395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2. 8; 424/88; 530/300, 395, 868

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

AUTOMATED PATENT SYSTEM, DIALOG DATABASE files 5, 155, 351, 399. Key words: autoimmun? Sprue, celiac disease, major histocompat?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Immunology, volume 145, number 6, issued September 15, 1990, A. G. Lamont, et al., "Inhibition of experimental autoimmune encephalomyelitis induction in SJL/J mice by using a peptide with high affinity for IA-s molecules", see pages 1687-1693, especially abstract.	1-14
Y	The Lancet, volume 1, issued 06 March 1976, A. S. Pena, et al., "HLA-DW3 associated with coeliac disease", pages 506-508, see entire document.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 JULY 1994	15 AUG 1994

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